Giant Lipid Vesicles Filled with a Gel: Shape Instability Induced by Osmotic Shrinkage

A. Viallat, J. Dalous, and M. Abkarian
Laboratoire de Spectrométrie Physique, Université J. Fourier, Saint Martin d'Hères, France

ABSTRACT We report the properties of giant lipid vesicles enclosing an agarose gel. In this system, the lipid bilayer retains some basic properties of biological membranes and the internal fluid exhibits viscoelastic properties, thus permitting us to address the question of the deformation of a cell membrane in relation to the mechanical properties of its cytoskeleton. The agarose gel (concentration $c_{0gel} = 0.07\%$, 0.18%, 0.36%, and 1% w/w), likely not anchored to the membrane, confers to the internal volume elastic moduli in the range of 10–10^4 Pa. Shapes and kinetics of de-swelling of gel-filled and aqueous solution-filled vesicles are compared upon either a progressive or a fast osmotic shrinkage. Both systems exhibit similar kinetics. Shapes of solution-filled vesicles are well described using the area difference elasticity model, whereas gel-filled vesicles present original patterns: facets, bumps, spikes ($c_{0gel} < 0.36\%$), or wrinkles ($c_{0gel} \geq 0.36\%$). These shapes partially vanish upon re-swelling, and some of them are reminiscent of echinocytic shapes of erythrocytes. Their characteristic size (microns) decreases upon increasing c_{0gel}. A possible origin of these patterns, relying on the formation of a dense impermeable gel layer at the vesicle surface and associated with a transition toward a collapsed gel phase, is advanced.

INTRODUCTION

Giant unilamellar vesicles are closed, fluid, and semipermeable soft shells. Their sizes and curvatures are similar to those of living cells and their lipid bilayer membrane exhibits basic properties of biological membranes (Menger and Angelova, 1998; Lipowsky and Sackmann, 1995). In particular, the lipid bilayer allows water permeation, leading to control of the swelling ratio and the deformability of the vesicles. It also allows the insertion of various membrane proteins (Roux et al., 2002) and/or adhesion molecules (see for example, Boulbitch et al., 2001). These features made them very attractive objects to develop physics closely linked to a number of questions in cellular biology. Giant unilamellar vesicles have been recently used as minimal systems allowing tubulation with molecular motors (Roux et al., 2002) or as simple models able to isolate, control, and modulate specific cell characteristics to enable us to understand physical mechanisms in situations close to life. However, their use remains restricted, due to the over-simplified structure of their internal volume. A first crucial limitation comes from the absence of cytoplasm and cytoskeleton in usual vesicles. For instance, although the well-known discoid shape of red blood cells well corresponds to an equilibrium shape for deflated vesicles (Seifert, 2000), it is difficult to experimentally handle giant discoid vesicles: they easily undergo scissions, and form threads or necklaces when they experience weak stresses such as, for instance, a small shear flow (Abkarian, 2002). An even more important example concerns the relationship between the complex mechanics of a cell, its properties of deformation, and its motility under an external field or under a flow. This problem obviously cannot be approached with vesicles filled only with water. It is therefore of great interest to confer controlled tougher mechanical properties to lipid vesicles. One way is to prepare vesicles whose membrane is partially polymerized or coated with a polymer. In an early work, Gaub et al. (1984) obtained vesicles with a bilayer containing a fraction of polymerized butadiene lipids. More recently, self-assembled networks of actin filaments were attached to the outer surface of giant unilamellar vesicles and the viscoelastic behavior of the coated membrane was clearly demonstrated (Helfer et al., 2001). In this case, however, the external layer of the membrane was not accessible because of the coating. An alternative way is to introduce a network into the internal volume of the vesicles. Due to the central role played by actin based-cytoskeleton, actin networks cross-linked by natural linkers such as α-actinin and filamin have been generated in giant vesicles (Haeckl et al., 1998; Limozin and Sackmann, 2002). Actin polymers were found to form isotropic networks of rigid filaments. Few attempts have been made to create vesicles containing more flexible polymer gels, although flexible cytoskeletons are found in nature as, for instance, in red blood cells. Very recently, two independent methods have been proposed to introduce a flexible network of poly(*n*-isopropylacrylamide) attached to the inner monolayer of liposomes (Stauch et al., 2002; Kazalov et al., 2002). Both studies were applied to small liposomes (<1 μm) so that direct observation by microscopy was not possible. The other problem was that the mechanical properties of these gels were not known.

The present work first focuses on the method of preparation of giant unilamellar lipid vesicles filled with a flexible polymer gel, whose elastic modulus can be controlled and adjusted. These giant vesicles (>10 μm) can

Submitted April 30, 2003, and accepted for publication November 4, 2003.

Address reprint requests to Dr. Annie Viallat, Laboratoire de Spectrométrie Physique, UMR 5588, Université J. Fourier, BP 87 38402 St. Martin d'Hères cedex, France. Tel.: 33-47-663-5645; E-mail: viallat@spectro.ujf-grenoble.fr.

0006-3495/04/04/2179/09 $2.00

be directly observed by phase contrast microscopy leading to an easy exploration of their behavior under various external fields. Secondly, it deals with the shapes presented by vesicles filled with gel, when shrinkage is induced by osmotic pressure.

Morphologies of vesicles filled with simple liquid have been extensively studied (Helfrich, 1973; Svetina et al., 1982; Miao et al., 1994; Lipowsky and Sackmann, 1995). When the reduced volume of partially deflated vesicles varies, a great variety of shapes (pears, peanuts, starfish. . .) is observed. Various models were developed to predict the shape behavior of vesicles. The most successful model is undeniably the area difference elasticity model (Miao et al., 1994; Mui et al., 1995; Döbereiner et al., 1997). In this model, the numbers of lipids in internal and external layers of the membrane can be different and the areas of the two layers are not fixed. These features result in an effective area difference between the two layers.

As a consequence, the morphology of vesicles is controlled by their reduced volume V and by an effective reduced area difference, which is determined by the spontaneous curvature of the Helfrich model and by a curvature-induced area difference between the inner and outer monolayers comprising the bilayer membrane.

The crucial feature concerning gel-filled vesicles is that the internal elastic gel prevents large deformations (pears, buds) and scissions induced by deflation from happening. The rubber elasticity of the gel is thought to govern the shape behavior of gel-filled vesicles under large osmotic shrinkage. This behavior may be complex since it has been long reported (Matsuo and Tanaka, 1988) and recently theoretically described (Maskawa et al., 1999) that gels submitted to a sudden strong volume transition may exhibit transient or permanent shape instabilities. In this study, we propose a first limited address of this question of morphology under osmotic shrinkage by showing various shapes exhibited by gel-filled vesicles under an osmotic pressure, applied either progressively or suddenly.

The article is organized as follows. We first report the method of preparation of vesicles filled either with simple liquid or with agarose gel. Then, we describe the shapes presented by simple vesicles upon a slow or sudden osmotic pressure change. Finally, we report observations of the shapes of gel-filled vesicles under osmotic de-swelling and a tentative explanation of the observed behavior is advanced.

MATERIALS AND METHODS

Preparation of lipids (DOPC) films

DOPC purchased from Sigma (St. Louis, MO) was dissolved in chloroform and methanol (9:1 volume ratio) at a concentration of 1.5 mg/ml. The solution of lipid was spread on two glass plates coated with indium tin oxide and the solvent was evaporated for 1 h under primary vacuum. Then, the two plates separated by a Teflon spacer (1-mm thick) were assembled to form a sealed chamber.

Electroformation of water-filled vesicles

The vesicles were prepared by the electroformation method (Angelova et al., 1992). The chamber was filled with a sucrose solution (50 mM). An AC voltage of 10 Hz was applied immediately to the ITO plates, and the voltage was progressively increased from 200 mV to ~1.2 V within 1 h and kept constant for 3 h. The frequency was finally decreased to 4 Hz with a voltage equal to 0.4–0.6 V to detach the vesicles from the glass plate.

Preparation of agarose solutions

Various agarose concentrations were prepared, above the gelation concentration, which is 0.05% w/w: 0.07% w/w, 0.18% w/w, 0.36% w/w, and 1% w/w. They were first homogenized at 100°C during at least 1 h. The solutions then slowly cooled down. When a temperature of ~60°C was reached, sucrose was added to the solutions (20 mM) to control the osmotic pressure of the vesicles. When kept above 50°C the solutions remained liquid during 20 min.

Electroformation of agarose-filled vesicles

The electroformation method was modified as follows: the chamber was filled with the solution of sucrose and agarose at 50°C. During the electroformation, the chamber and the tools (syringes, solution bottles) were kept at 50°C using a flow of warm air directed toward the platinum of the microscope to maintain the agarose solution in the sol state. The electroformation time was limited to 18 min to avoid agarose gelation. At the frequency of 10 Hz, the voltage was first increased from 0.2 V to 0.5 V within 5 min, then to 1 V within the next 5 min, and finally to 1.2 V. After 17 min the frequency was lowered to 4 Hz to detach the vesicles from the substrate. The vesicle solution was then gently injected into a large volume (from 4 to 20 ml) of an isoosmolar glucose solution (20 mM) kept at 50°C. The agarose concentration of the solution in which the vesicles were in suspension was then far below the gelation concentration. Finally, the solution containing the vesicles was set at 4°C, allowing gelation of agarose within the vesicles only.

Osmotic de-swelling: preparation of the chamber

To apply various controlled osmotic pressures to the vesicles, a small tank inspired from that reported in Bernard (1999) was made. A first small cylindric plastic ring (2.5-mm thick, central cavity of diameter equal to 20 mm), which determined an internal volume equal to 785 μL, purchased from Merck Eurolab, Dorset, UK (ref. 26704), was fixed on a coverslip to create a small tank. It was first half-filled (393 μL) with a 20 mM glucose solution. It was then fully filled by injecting 393 μL of the solution containing the vesicle suspension. Vesicles settled to the bottom. An inorganic filter disc with pores of 0.2-μm diameter (Anodisc membranes purchased from Merck Eurolab, ref. 69908) was set at the top of the tank. Four cylindric plastic rings of the same model were set above the initial one to increase the tank volume and were filled with a hyperosmolar glucose solution (Fig. 1) to create an osmotic stress. The concentration of the glucose solution was chosen according to the osmotic pressure we wanted to apply. The filter disc allowed for smooth diffusion of glucose toward the settled vesicles and limited convection currents. When a strong and fast osmotic pressure was wanted, the internal volume defined by the four rings were filled in one time. When a slow progressive osmotic pressure was wanted, the rings were added and filled one by one by glucose solutions of increasing osmolarities (increments of ~10 mM). All concentrations were known with an experimental error of 4%. The timescale for equilibration in the chamber was difficult to estimate because of the small convection currents that exist during tank filling (one could observe a slow motion of the vesicles over a distance of a few vesicle diameters during and after tank filling). It was experimentally estimated by following the kinetics of shrinkage of several

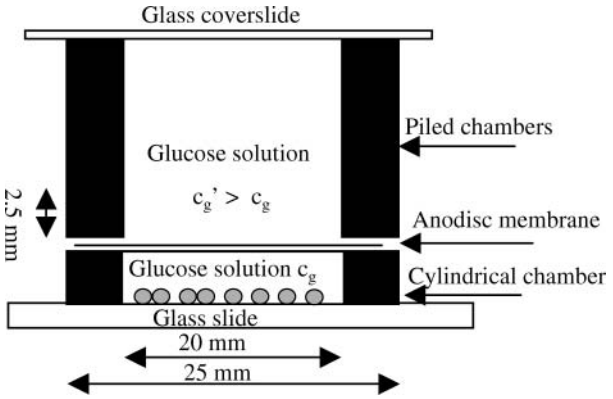

FIGURE 1 Experimental setup for application of osmotic shocks.

vesicles under various osmotic shocks. Stable volumes and shapes of vesicles were reached after a maximal time of 40 min. This delay was always observed between the filling of two successive rings to ensure homogenization of the solution. Kinetics of slow and fast de-swelling were observed during ~5 h and 2 h, respectively.

Observations were made using a phase contrast inverted microscope (Leica DMB, Leica, Solms, Germany) and a charge-coupled device camera (Cohu 4910, Cohu, San Diego, CA). Image analysis was performed using an image software (NIH, 1.62c).

RESULTS

Vesicles filled with sucrose solution under osmotic de-swelling

To study the role of entrapped gel on the behavior of vesicles, similar experiments were carried out on vesicles filled with simple sucrose solution and on vesicles filled with gel. In the first section, we report on the behavior of vesicles filled with a sucrose solution (50 mM) upon osmolyte addition on the outside. Two types of experiments were performed: 1), slow shrinkage was induced by stepwise increase of the concentration of the outer glucose solution (final outer glucose concentration, 162 mM); and 2), fast shrinkage by sudden addition of 400 mM glucose above the anodisc membrane (see Fig. 1), which led to a final glucose concentration of 330 mM after equilibration of the concentration gradient.

Step-by-step progressive shrinkage

We followed the shrinkage of several vesicles during the whole process (5 h). Starting from quasispherical shapes, the vesicles generally strongly deformed with time. Budding and scission of a deflated vesicle into two smaller spherical daughters were sometimes observed.

Osmotic pressure

One vesicle remained spherical during the shrinkage and its radius decreased progressively from 25 μm to 17 μm, which corresponds to a loss of ~50% of the vesicle area. Such a behavior was already observed by Boroske et al. (1981) under osmotic pressure and by Sandre et al. (1999) on DOPC vesicles marked with a membrane-bound fluorescent probe under a strong continuous illumination. The associated decrease of the surface area of the vesicle was interpreted in both cases as a loss of lipids from the vesicle membrane. Sandre, using fluorescence microscopy, observed some small fluorescent particles close to de-swelling vesicles that he interpreted in terms of a concentrated lipid superstructure (bicontinuous cubic phase; Kummrow and Helfrich, 1996; Klösgen and Helfrich, 1997), formed by the collapse of the excess of membrane. These lipid wells might be involved in the behavior observed in this work, the transition being possibly associated with the strong applied osmotic pressure, since neither significant budding nor daughter vesicles were observed using phase contrast microscopy.

Time-sequence images allowed the determination of the volume of the vesicle at each step. A final decrease of two-thirds of the initial volume was obtained. By assuming that the number of sucrose molecules present in the inner vesicle volume remained constant, the sucrose concentration of the inner solution and the corresponding osmotic pressure were derived at each of the four shrinkage stages. As it is clearly seen in Fig. 2, the osmotic pressures inside and outside the vesicle are equal, thus disclosing that the osmotic equilibrium was reached at the end of each stage.

Shapes

A great variety of vesicle shapes were obtained in the chamber at the end of the shrinkage process: ellipsoids, dumbbells, stomatocytes, discocytes, pears, stars, etc. The typical shapes, shown in Fig. 3, are characteristic of the main morphologies predicted by the area difference elasticity model (Mui et al., 1995). Such variety of shapes has been obtained when the area difference was monitored by temperature change (Döbereiner et al., 1993; Käs and Sackmann, 1991). In our case, we observed, at the same time in the same chamber, vesicle shapes corresponding to various reduced volumes probably resulting from uncontrolled vesicle

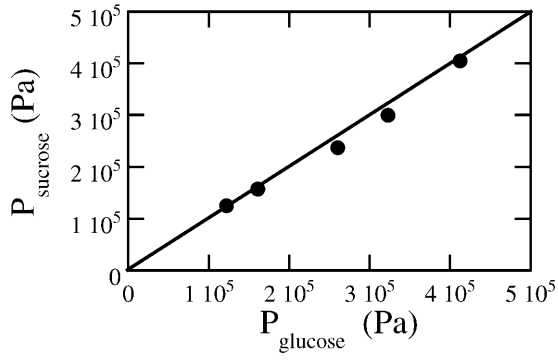

FIGURE 2 Step-by-step osmotic shrinkage of vesicles filled with sucrose solution: inner sucrose osmotic pressure versus outer glucose osmotic pressure.

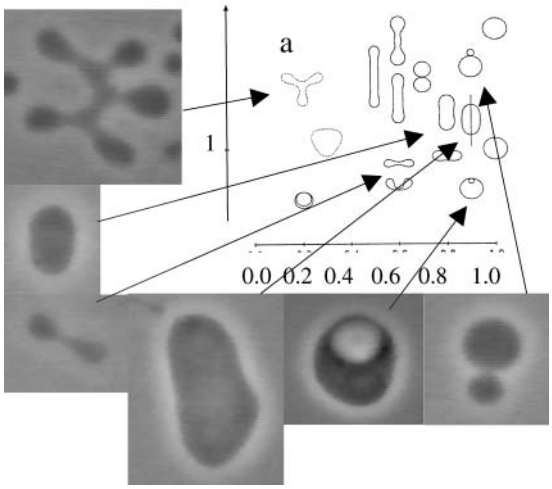

FIGURE 3 Main shapes observed on vesicles filled with sucrose solution under step-by-step osmotic shrinkage. Comparison with the shapes predicted from the area difference elasticity model.

scissions and various membrane area differences due to an asymmetry between the two layers of the membrane.

Fast shrinkage

Many vesicles were imaged at the end of the shrinkage process and three vesicles were monitored during the whole de-swelling process (2 h). Scissions occurred in two of these vesicles; one remained spherical. As observed for the step-by-step shrinkage process, vesicles were generally strongly deformed.

Shapes

As in the case for slow shrinkage, we observed various shapes. We, however, observed more vesicles deformed on a very small scale, such as 1), the formation of multiple small invaginations of the membrane pointed toward the interior of the vesicles, which looked like raspberries; and 2), the formation of lipid pearl necklaces (Fig. 4). The formation of necklaces was probably favored by the appearance of small currents in the observation chamber induced by the addition of a large amount of concentrated glucose solution. Due to the flow, the rear of the vesicles lengthened (Abkarian et al., 2002), leading to a local destabilization of the membrane, which generated necklaces.

Vesicles filled with agarose gel

In this section, we study the behavior of vesicles filled with agarose gel. We first shortly recall the properties of agarose gels that are useful for preparation and characterization of gels within vesicles. Then we briefly describe our observations on gel-filled vesicles at osmotic equilibrium. We report the results of experiments of osmotic shrinkage, which were carried out in the same way as that performed on simple vesicles.

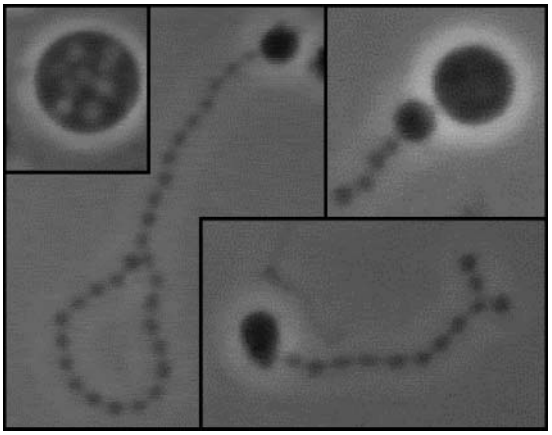

FIGURE 4 Main shapes observed on vesicles filled with sucrose solution under fast osmotic shrinkage.

Agarose gels

The melting temperature of agarose chains in dilute water solution is close to 100°C. Below this temperature, the system becomes progressively semicrystalline and bundles of double-crystalline helices are formed, which play the role of cross-links. This leads to the formation of a thermoreversible gel, when the agarose concentration is higher than a critical concentration of ~0.05% w/w (Arnott et al., 1974). The kinetics of gelation is controlled by a nucleation-based process, in which the rate-limited step (helix-helix aggregation) depends on temperature. At $T = 50$°C, gelation takes place within 30 min, whereas at 5°C, it occurs within a few minutes. This property allows for sufficient time to electroform vesicles encapsulating fluid agarose solution at 50°C and to dilute the external solution below the gel critical-concentration (to avoid gelation of the external solution in which vesicles are in suspension). The structure of these gels can be pictured as a packing of rigid bunches of helices. Their elastic moduli depend on the concentration: they increase from 10 to 10^4 Pa when increasing the agarose concentration from 0.07 to 1% w/w. From a given initial concentration of c^0_{aga}, the gels can be reversibly de-swollen and re-swollen if the degree of de-swelling is not too great ($c_{aga}/c^0_{aga} < 5$). Observations of the de-swelling of agarose gels, performed under uniaxial conditions, reported that an isotropic-anisotropic phase transition is observed from the isotropic swollen state of preparation to a concentrated state that is strongly bi-refringent and elastically anisotropic (Rochas et al., 1996, 1997).

Gel-filled vesicles: isoosmolar conditions

The vesicles filled with agarose gel and sucrose solution were diluted into an isoosmolar glucose solution. They were observed using phase contrast microscopy at room temperature. They appeared slightly marbled and presented spherical or quasispherical shapes, with radii ranging from

10 to 35 μm. Thermal fluctuations were visible at high magnification (60×) on some vesicles, thus suggesting that the coupling between the gel and the membrane is not strong.

Step-by-step de-swelling

We monitored the step-by-step de-swelling process of several vesicles, prepared with an initial agarose concentration of 0.18% w/w, 0.36% w/w, and 1% w/w. Vesicles were first diluted into 20 mM. Then, the concentration of glucose was increased by addition of a more concentrated glucose solution in three or four stages of at least 40 min each, up to a final glucose concentration ranging from 90 to 154 mM. In a typical experiment, the outer glucose concentration was for each stage equal to 20 mM, 35 mM, 56.6 mM, 117.5 mM, and 154 mM, respectively. As detailed below, the shrinkage ratio of vesicles depended on their initial agarose concentration and on the applied external pressure.

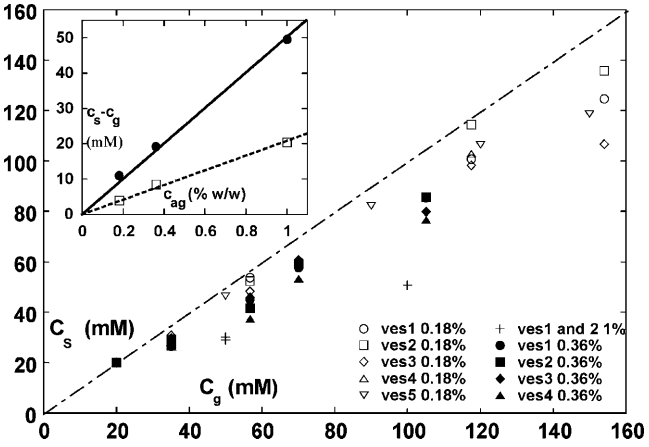

FIGURE 5 Variations of the sucrose concentration c_s in vesicles filled with agarose gel versus the external glucose concentration c_g. (*Insert*) Variation of $\Delta = c_s - c_g$ observed for two values of c_g (50 mM and 100 mM) with the initial agarose concentration.

Osmotic pressure

Starting from an initial volume of V_0, we estimated the volume V of each vesicle at the different stages of shrinkage. We first determined the contour of the vesicle, then we found its center of mass and we performed up to 36 measurements of radii from this center of mass, by successive rotations of 10°. This yielded an average vesicle radius. We determined the volume of the equivalent sphere, assuming axisymmetric shapes. Although the error bar was high for the most deformed vesicles, this calculation gave the right order of magnitude of the volume. From this value, we derived concentrations of agarose and sucrose by assuming no sucrose or glucose leak out of or into the system.

Typical variations of the sucrose concentration in vesicles, c_s, with the external glucose concentration, c_g, are shown in Fig. 5. The straight line corresponds to the curve $c_s = c_g$. All experimental points are below this curve, showing that the internal sucrose concentration is lower than the external glucose concentration. The results are different from those presented in Fig. 2 for vesicles filled with a sucrose solution, where inner and outer osmotic pressures and external glucose and internal sucrose concentrations were equal. This result shows that under the same external pressure, the inner sucrose concentration of gel-filled vesicles is lower than that of solution filled-vesicles: the gel retains water inside the vesicle. This water retention effect is clearly illustrated by plotting the variation of the difference $\Delta = c_g - c_s$ versus the initial agarose concentration, c^0_{aga} (Fig. 5, *insert*) for two values of the external glucose concentration, $c_g = 50$ mM and $c_g = 100$ mM, respectively. The observed variations show that the higher the initial agarose concentration c^0_{aga} and the external glucose concentration, the more important the water retention effect of the gel. To better understand the origin of this effect, let us examine the different physical contributions to the osmotic pressures exerted by the external glucose solution, P_{glu}, and by the inner solution of the vesicle, P_{gel}, which are equal at equilibrium. The pressure P_{gel} is obtained from the derivative of the total free energy of the gel with respect to the number of solvent molecules.

It is classically written for swollen rubbers as the sum of a mixing free energy corresponding to the osmotic interactions between chains and a three-dimensional elastic retraction contribution to the free energy (having the same entropic origin as the uniaxial modulus of a rubber). Regarding this latter contribution, the elastic response to a deformation of classical polymer gels implies stretching of chain segments and rearrangements of the network on a larger scale than the mesh size (Flory, 1953; Dusek and Prins, 1969; Mark and Erman, 1988). For the simplest classical gels (long flexible chain segments, chain deformation in affinity with the macroscopic strain), the pressure exerted by a swollen gel is written by Bastide and Candau (1996) as $P_{gel} = P_{sol} + P_{el} = P_{sol} - G$, where P_{sol} is the osmotic pressure of the un-cross-linked polymer solution, P_{el} is the elastic pressure, and G is the gel shear modulus.

In our case, the mixing term involves a ternary solution with water, sucrose, and agarose chains. However, the osmotic contribution of agarose chains has been shown to play no significant role in the equilibrium state of agarose gels swollen in water (Rochas et al., 1996, 1997). The mixing term is therefore dominated by the osmotic contribution of sucrose, P_{mix}. Rochas and co-workers have shown, by experiments of shrinkage of swollen agarose gels, that P_{el} was quantitatively equal to G, as in the classical rubber theory of gels (although the reasons could be different). Consequently, by writing equilibrium conditions $P_{glu} = P_{gel}$ and $P_{gel} = P_{mix} + P_{el}$, and replacing P_{el} by G, we obtain $P_{glu} - P_{mix} = G$. The glucose osmotic pressure in solution with water is proportional to the glucose concentration in the outer solution. The term P_{mix}, however, even

though it is dominated by the sucrose contribution, may slightly differ from the sucrose osmotic pressure in solution with pure water, due to possible small interaction of sucrose with agarose. The deviation is supposed to be small (and linear with the concentration) in the low range of concentrations we studied, but to avoid ambiguity, we preferred to use $\Delta = c_g - c_s$, to reflect the gel shear modulus behavior rather than the difference between osmotic pressures in pure water of external glucose and internal sucrose. The parameter Δ, like G, should therefore depend on the one hand on the structural features of the network, like the cross-link ratio, determined by the initial agarose concentration c^0_{aga}, and on the other hand, on its deformation (de-swelled) state with respect to its initial state, characterized by the ratio V_0/V. By plotting the variation of Δ versus these two parameters in a double logarithmic representation (Fig. 6), we observe that the data fall well on a single curve of variation of Δ versus the agarose concentration in the vesicle, c^0_{aga}, V_0/V, with a slope equal to 2. This result shows that the elastic response of the gel varies with the square of the concentration, $(c^0_{aga}, V_0/V)^2$, in the displayed range of external pressures. Such power laws with exponents close to 2 have been shown for several biopolymers, where the polymer concentration corresponds to that of gel preparation (Normand et al., 2000). It was advanced that the exponent 2 may reflect the kinetic order of the mechanism of gel formation. In our case, however, the gel agarose concentration in not that of gel formation; it results from the de-swelling process, whose effect on the reorganization of the gel structure is not known. It is worth noting that for very small external pressure no gel deformation was observed and, for high external pressures, the maximal volume reduction we could obtain was of the order of 80%.

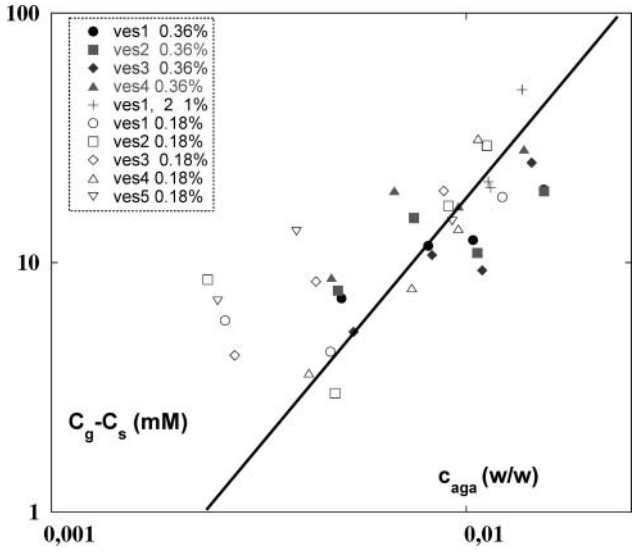

FIGURE 6 Log-Log variation of the parameter $\Delta = c_g - c_s$ versus the agarose concentration in the vesicles. Straight line is a fit with a slope equal to 2.

Shapes

The highest shrinkage ratios V_o/V were obtained for vesicles prepared with the lowest agarose concentration. Moreover, irregular shapes were observed upon increasing the external glucose concentration. At the beginning of the process, for moderate external glucose concentrations, small shrinkages were observed and vesicle shapes remained quasispherical. Then, when the concentration of the external glucose solution increased, shape instabilities were observed. In particular, vesicles prepared with an agarose concentration of 0.18% (w/w) presented facets. For even higher external glucose concentrations, these vesicles strongly shrunk and presented very irregular shapes with formation of bumps and spikes, as shown on one vesicle in Fig. 7. Vesicles prepared with an initial agarose concentration of 0.36% and 1% (w/w) less shrunk but also presented small-scale shape deformation. The typical sizes of bumps and spikes were estimated to range between ~0.5 and 3 μm, the biggest spikes being observed on the most strongly deformed vesicles (initial agarose concentration of 0.18% w/w). Deformations were at a smaller scale when the elastic modulus of the gel was higher (0.36 and 1% w/w). The characteristic distance between adjacent peaks on a vesicle were found in the range of 2–8 μm, depending on the observed vesicles.

Fast shrinkage

The effect of fast shrinkage was studied on vesicles prepared with an initial agarose concentration of 0.07% w/w, using three methods for osmolyte addition.

In the first method, vesicles were dipped in a hyperosmolar glucose solution. The observation started after 10 min, which was the time necessary for vesicles to settle at the bottom of the chamber. Inasmuch as the osmotic shock was strong, the

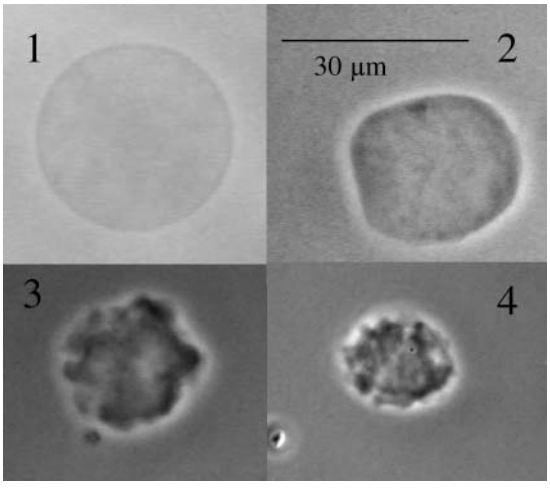

FIGURE 7 Successive shapes presented by a gel-filled vesicle (initial concentration of agarose 0.18% w/w, initial radius 31.1 μm) under osmotic shrinkage in four steps (with *1* as *initial state* and *4* as *final state*).

vesicles were already much deformed at the beginning of the observations: they presented large spikes of well-defined sizes.

In the second method, we used a large chamber in which a highly concentrated glucose solution was gently injected close to the vesicles. The shrinkage of each vesicle was then observed. Upon shrinking, the membrane could sometimes separate from the gel and made threads and necklaces. The advantage of this method was that it enabled us to redilute the solution by injection of pure water. Vesicles re-swelled, and the spikes disappeared. The process was completely reversible if the deformation was not too large. If the volume reduction was ~80% of the initial volume, a small deformation remained, indicating a plastic regime.

In the third method, we proceeded as we did for vesicles filled with the simple sucrose solution. This method permitted us to observe the kinetics of de-swelling of four vesicles prepared with the agarose concentration of 0.07% w/w. Final concentrations of the external glucose solutions were either 324 mM (for three vesicles) or 404 mM (for one vesicle). Kinetics of de-swelling are shown in Fig. 8. It is clearly seen that all the displayed curves present the same slope during the first 300 s as that observed for vesicles without gel. This result shows that the presence of the gel does not change the dynamics of vesicle de-swelling. To understand this point, it is interesting to examine the different timescales involved in the de-swelling process. Concerning the gel, the kinetics of de-swelling of classical gels is generally considered to be controlled by the cooperative diffusion coefficient D (ratio of the gel osmotic longitudinal modulus to the mutual friction coefficient between polymer and solvent) and by the gel size (Tanaka and Fillmore, 1979; Matsuo and Tanaka, 1992). The associated characteristic time τ is equal to $R^2/\pi^2 D$ (the gel radius changes rapidly for times smaller than τ). In the systems studied here ($R =$ 10 μm), times τ are short (1 s for a diffusion coefficient as small as 10^{-7}/cm^2 per s). Moreover, agarose gels form rigid matrix, in which the polymer chains undergo no motion. Therefore, regarding the transport properties, the gel probably behaves as a porous medium of mesh size much larger than water molecules, so that water diffusion is expected to be of the order of bulk diffusion (10^{-5}/cm^2 per s). Finally, the characteristic time of gel de-swelling is then expected to be much shorter than the time of vesicle shrinking reported in Fig. 8, which is probably fixed by transport through the membrane.

As an exception, the de-swelling of one vesicle was faster within a time window of ~1 min. It is believed that during this time a pore was opened in the membrane, leading to a fast ejection of water, as can be seen in Fig. 8. This phenomenon was associated with a fast shrinkage localized on one side of the vesicle, which likely corresponded to the place where the pore opened.

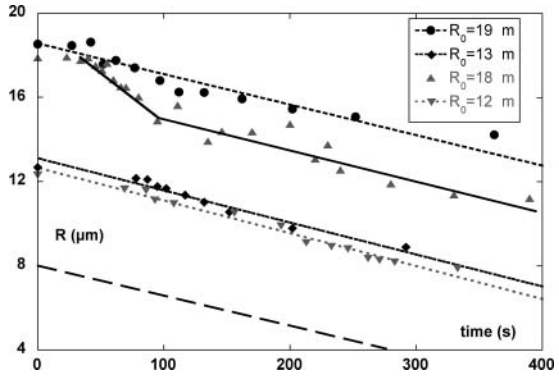

FIGURE 8 Kinetics of shrinkage of gel-filled vesicles (initial agarose concentration 0.07% w/w). Variation of average radii with time. The slope of the dotted line is that found for the kinetics of shrinkage of the vesicle filled with sucrose solution in Fig. 4.

Shapes

The observed shapes were very much deformed. Typical shapes are shown in Fig. 9. As observed in the case of step-by-step de-swelling for 0.18% w/w agarose gels, spherical vesicles progressively exhibited facets (for a volume reduction of ~10–15%), whereas spikes appeared at higher volume reductions. The maximum volume reduction was similar to that observed with vesicles filled with gels of initial concentration 0.18% w/w, i.e., of the order of 80%. Under these conditions, pronounced spikes were seen (see last picture of Fig. 9).

DISCUSSION AND CONCLUSION

We observed that shapes under osmotic shrinkage of gel-filled vesicles and of vesicles filled with sucrose solution are markedly different, although their kinetics of de-swelling is similar, i.e., solely limited by water transport through the membrane. Under comparable osmotic pressure conditions, gel-filled vesicles were seen to retain more water than simple vesicles. Such altered swelling property originates from the mechanical and structural properties of the agarose network. It cannot be understood simply in terms of a trivial osmotic contribution of agarose but involves an elastic pressure exerted by the gel. This effect is governed by a unique parameter, the agarose volume fraction, and is independent of the initial (before shrinkage) agarose concentration. Associ-

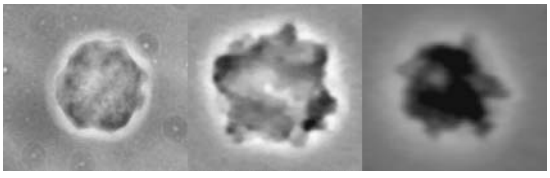

FIGURE 9 Typical shapes of vesicles filled with agarose gel (0.07% w/w) after fast osmotic shock.

ated vesicle shapes depend on the initial agarose concentration. Vesicles prepared with a low agarose concentration, i.e., with a gel of low elastic modulus, exhibit shape instabilities, facets, and well-defined sharp spikes whose characteristic size is of several microns. Vesicles prepared with higher agarose concentrations, i.e., with a gel of higher elastic modulus, presents smaller deformations (μm-size wrinkles). The observed shapes are stable with time but can vanish upon reswelling, although some residual deformations are still observed on the most deformed vesicles.

Pattern formation has long been reported in the literature in structures containing elastic networks, like cells and polymer gels. It has been known for more than 50 years (Ponder, 1948) that a number of physical and chemical agents can modify erythrocyte discoid shape, inducing a series of crenated echinocyte shapes characterized by micron-size spicules. Regular patterns are observed on polymer gels, when they undergo a volume phase transition upon heating, with solvent composition or osmotic pressure being an external parameter (Matsuo and Tanaka, 1992). Bamboo and bubbles patterns with alternate swollen and shrunken portions are observed, which are often stable for months.

Regarding erythrocyte, the full stomatocyte-discocyte-echinocyte sequence has been convincingly described (Mukhopadhyay et al., 2002; Lim et al., 2002) from the properties of its composite membrane (two-dimensional elastic spectrin network anchored in the plasma membrane). The model shows, as first advanced by Sheetz and Singer (1974), that shape modification at constant volume and area can be driven by changes in the relaxed area difference between the two leaflets of the membrane (bilayer-couple hypothesis) and by accounting for the stretch and shear elasticity of the spectrin network, as recently suggested (Iglic, 1997).

Although the shapes of gel-filled vesicles are strongly reminiscent of echinocytes, their structure (weak membrane-gel interactions, gel filling the whole vesicle volume) and the conditions of pattern formation (strong volume reduction for gel-filled vesicles) are different. For instance, according to this model, the length scale (which fixes spikes' size), defined as the square root of the ratio of the membrane-bendin rigidity with respect to the shear modulus, should be nanometric in our systems, contrary to observations. Moreover, we think that the membrane does not play the prominent role for shape control of shrinking gel-filled vesicles, since when the membrane separated from the gel upon deswelling and formed necklaces, spikes were still observed in the inner gel.

Pattern formation in strongly shrinking polymer gels was explained by Matsuo and Tanaka (1992) by formation of a dense impermeable layer at the gel boundary, where shrinkage starts. This step is followed by a change of the shape of the boundary, due to the flexibility of the outer impermeable layer, thus leading to permanent patterns. In agarose gel, a phase transition was already observed under uniaxial pressure or under slow drying from the gel top surface: in the upper gel region, a de-swollen, bi-refringent, elastically anisotropic state of low permeability was formed, which coexisted in stable equilibrium with the isotropic swollen phase in the lower gel part (Rochas et al., 1996, 1997). A similar process may occur in agarose-filled vesicles. A tentative explanation for the behavior reported here may be advanced in terms of osmotic shrinkage leading to the formation of a dense impermeable gel layer close to the surface, which stops further water expulsion, so that the subsequent deformation of the outer layer gives rise to pattern formation, in a way similar to that observed by Matsuo and Tanaka (1992).

Turning back to the shapes that a composite membrane (lipid bilayer supported by an elastic network) can present, the preparation of gel-filled vesicles reported in this work opens the way to the preparation of lipid vesicles enclosing a thin layer of a polymer gel anchored to the inner part of the lipid membrane. In this class of systems, the crucial physical parameters of membrane spontaneous curvature, mechanical properties of the quasi-two-dimensional network, and membrane/network coupling could be varied over a large range, contrary to red blood cells, by changing lipid compositions, cross-link densities, and anchoring molecule concentration, respectively. This class of system would be very appropriate to experimentally explore and test the shapes predicted from the bilayer-couple hypothesis (with biomechanics accounting for elastic terms), over a wide range of values of the parameters cited above. It would then allow us to get into the question of the role of specific biochemical probes on these shape control parameters. Such vesicles, based on flexible covalent polymer networks, are currently in preparation.

We thank C. Rochas for fruitful discussion and H. G. Döbereiner for his very helpful comments on our manuscript.

REFERENCES

Abkarian, M. 2002. Dynamique et formes d'enveloppes déformables sous champs externs: vésicules et globules rouges. PhD thesis. Université Joseph Fourier, Grenoble, France.

Abkarian, M., C. Lartigue, and A. Viallat. 2002. Tanktreading and unbinding of deformable vesicles in shear flow: determination of the lift force. *Phys. Rev. Lett.* 88:8103–8107.

Angelova, M. I., S. Soléau, P. Méléard, J. Faucon, and P. Bothorel. 1992. Preparation of giant vesicles by external AC electric fields. *Progr. Coll. Pol. Sci.* 89:127–133.

Arnott, S., A. Fulmer, W. E. Scott, I. C. Dea, R. Moorhouse, and D. A. Rees. 1974. The agarose double helix and its function in agarose gel structure. *J. Mol. Biol.* 90:269–284.

Bastide, J., and S. J. Candau. 1996. Structures of gels investigated by static scattering techniques. *In* Physical Properties of Polymer Gels. J. P. Cohen Addad, editor. John Wiley and Sons, New York.

Bernard, A.-L. 1999. Perméabilité de bicouches lipidiques vésiculaires soumises à une déformation: perceuse moléculaire et autres exemples. PhD thesis, Université Pierre et Marie Curie, Paris, France.

Boroske, E., M. Elwenspoek, and W. Helfrich. 1981. Osmotic shrinkage of giant egg-lecithin vesicles. *Biophys. J.* 34:95–109.

Boulbitch, A., Z. Guttenberg, and E. Sackmann. 2001. Kinetics of membrane adhesion mediated by ligand-receptor interaction studied with a biomimetic system. *Biophys. J.* 81:2743–2751.

Döbereiner, H. G., J. Käs, D. Noppl, I. Sprenger, and E. Sackmann. 1993. Budding and fission of vesicles. *Biophys. J.* 65:1396–1403.

Döbereiner, H. G., E. Evans, M. Kraus, U. Seifert, and M. Wortis. 1997. Mapping vesicle shapes into the phase diagram: a comparison of experiment and theory. *Phys. Rev. E.* 55:4458–4474.

Dusek, K., and W. Prins. 1969. Structure and elasticity of non-crystalline polymer networks. *Adv. Pol. Sci.* 6:1–102.

Flory, P. J. 1953. Principles of Polymer Chemistry. Cornell University Press, Ithaca, NY.

Gaub, H., E. Sackmann, R. Buschl, and H. Ringsdorf. 1984. Lateral diffusion and phase separation in two-dimensional solutions of polymerized butadiene lipid in dimyristoylphosphatidylcholine bilayers. A photobleaching freeze fracture study. *Biophys. J.* 45:725–731.

Haeckl, W., M. Baermann, and E. Sackmann. 1998. Shape changes of self-assembled actin bilayer composite membranes. *Phys. Rev. Lett.* 80: 1786–1789.

Helfer, E., S. Harlepp, L. Bourdieu, J. Robert, F. C. MacKintosh, and D. Chatenay. 2001. Viscoelastic properties of actin-coated membranes. *Phys. Rev. E.* 63:021904.

Helfrich, W. 1973. Elastic properties of lipid bilayers: theory and possible experiments. *Z. Naturforsch.* 28c:c693–c703.

Iglic, A. 1997. A possible mechanism determining the stability of spiculated red blood cells. *J. Biomech.* 30:35–40.

Käs, J., and E. Sackmann. 1991. Shape transitions and shape stability of giant phospholipid vesicles in pure water induced by area-to-volume changes. *Biophys. J.* 60:825–844.

Kazalov, S., M. Kaholek, I. Teraoka, and K. Levon. 2002. UV-gelation on nanometer scale using liposome reactor. *Macromolecules.* 35:1911–1920.

Klösgen, B., and W. Helfrich. 1997. Cryo-transmission electron microscopy of a superstructure of fluid dioleylphosphatidylcholine (DOPC) membranes. *Biophys. J.* 73:3016–3029.

Kummrow, M., and W. Helfrich. 1996. Collapse of giant phosphatidylcholine vesicles. *Chem. Phys. Lipids.* 79:147–156.

Lim, G. H. W., M. Wortis, and R. Mukhopadhyay. 2002. Stomatocyte-discocyte-echinocyte sequence of the human red blood cell: evidence for the bilayer-couple hypothesis from membrane mechanics. *Proc. Natl. Acad. Sci. USA.* 99:16766–16769.

Limozin, L., and E. Sackmann. 2002. Polymorphism of cross-linked actin networks in giant vesicles. *Phys. Rev. Lett.* 89:16.

Lipowsky, R., and E. Sackmann. 1995. Structure and dynamics of membranes. *In* Handbook of Biological Physics. Elsevier/North-Holland, The Netherlands.

Mark, J., and B. Erman. 1988. Rubberlike Elasticity, a Molecular Prime. John Wiley, New York.

Maskawa, J. I., T. Takeuchi, K. Maki, K. Tsujii, and T. Tanaka. 1999. Theory and numerical calculation of pattern formation in shrinking gels. *J. Chem. Phys.* 110:10993–10999.

Matsuo, E. S., and T. Tanaka. 1988. Kinetics of discontinuous volume-phase transition of gels. *J. Chem. Phys.* 89:1695–1703.

Matsuo, E. S., and T. Tanaka. 1992. Patterns in shrinking gels. *Nature.* 358:482–485.

Menger, F. M., and M. I. Angelova. 1998. Giant vesicles: imitating the cytological processes of cell membranes. *Acc. Chem. Res.* 31:789–797.

Miao, L., U. Seifert, M. Wortis, and H. G. Döbereiner. 1994. Budding transitions of fluid-bilayer vesicles: the effect of area-difference elasticity. *Phys. Rev. E.* 49:5389–5407.

Mui, B. L. S., H. G. Döbereiner, T. D. Madden, and P. R. Cullis. 1995. Influence of transbilayer area asymmetry on the morphology of large unilamellar vesicles. *Biophys. J.* 69:930–941.

Mukhopadhyay, R., G. H. W. Lim, and M. Wortis. 2002. Echinocyte shapes: bending, stretching, and shear determine spicule shape and spacing. *Biophys. J.* 82:1756–1772.

Normand, V., D. L. Lootens, E. Amici, K. P. Plucknett, and P. Aymard. 2000. New insight into agarose gel mechanical properties. *Biomacromolecules.* 1:730–738.

Ponder, E. 1948. Hemolysis and Related Phenomena. Grune and Stratton, New York.

Rochas, C., A. M. Hecht, and E. Geissler. 1996. Swelling properties of agarose gels. *J. Chem. Phys.* 93:850–857.

Rochas, C., A. M. Hecht, F. Horkay, and E. Geissler. 1997. Anisotropic agarose gels. *Macromol. Symp.* 114:173–178.

Roux, A., G. Capello, J. Cartaud, J. Prost, G. Goud, and P. Bassereau. 2002. A minimal system allowing tubulation with molecular motors pulling on giant liposomes. *Proc. Natl. Acad. Sci. USA.* 99: 5394–5399.

Seifert, U. 2000. A theoretical perspective. *In* Giant Vesicles. P. L. Luisi and P. Walde, editors. John Wiley and Sons. Chichester, England.

Sandre, O., L. Moreaux, and F. Brochard-Wyart. 1999. Dynamics of transient pores in stretched vesicles. *Proc. Natl. Acad. Sci. USA.* 96: 10591–10596.

Sheetz, M. P., and S. J. Singer. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA.* 71:4457–4461.

Stauch, O., T. Uhlmann, M. Fröhlich, R. Thomann, M. El-Badry, Y.-K. Kim, and R. Schubert. 2002. Mimicking a cytoskeleton by coupling poly(*n*-isopropylacrylamide) to the inner leaflet of liposomal membranes: effects of photopolymerisation on vesicle shape and polymer architecture. *Biomacromolecules.* 3:324–332.

Stauch, O., R. Schubert, G. Savin, and W. Burchard. 2002. Structure of artificial cytoskeleton containing liposomes in aqueous solution studied by static and dynamic light scattering. *Biomacromolecules.* 3:565–578.

Svetina, S., A. Ottova-Leitmannova, and R. Glaser. 1982. Membrane bending energy in relation to bilayer couples concept of red blood cell shape transformations. *J. Theor. Biol.* 94:13–23.

Tanaka, T., and D. J. Fillmore. 1979. Kinetics of swelling of gels. *J. Chem. Phys.* 70:1214–1218.